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Order of Authors:	R. Alex Speers, B.Sc.(AGr.), M.Sc., Ph.D.
	Dawn L. Maskell
	Maria E. Josey

The Impact of Artificially Induced Respiratory Deficient Yeast on Beer Flavour and Fermentation

Maria Josey[1], Dawn L. Maskell[1], R. Alex Speers[1,2*]

[1]International Centre for Brewing and Distilling, Institute of Biological Chemistry, Biophysics and Bioengineering, School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, UK

[2]Canadian Institute of Fermentation Technology, Dalhousie University, Halifax, CAN

Corresponding author: R. Alex Speers
Canadian Institute of Fermentation Technology/ PEAS
1360 Barrington Street, Rm. D318 A.L MacDonald Building •
Sexton Campus, Dalhousie University
P.O Box 15000, Halifax, NS
B3H 4R2 Canada
Tel: 902.494.3146
Alex.Speers@dal.ca

Abstract

Respiratory deficient cells or 'petites' are the most common type of mutation found in brewing yeast. Very high levels of petites are known to contribute to unwanted flavours in beer along with yeast flocculation problems during fermentation. However, little is known about the impact of petites when present at naturally occurring frequencies. Accordingly, this study investigated if petites – present at low frequencies – affect beer flavour and fermentation profiles. Laboratory [20 mL] fermentations were undertaken with yeast that contained a range of petite populations 3.7, 5.1, 8.7, and 10.8 %). During fermentation, the yeast in suspension, wort density, and alcohol were monitored. At the end of the fermentation, the beer was analysed for volatile flavour compounds. Correlations between petite levels and levels of vicinal diketones, acetate esters and medium chain fatty acid (MCFA) ethyl esters existed. Higher alcohol levels were unchanged (propan-1-ol, 3-methyl butanol, 2-methyl butanol, and isobutanol) with increasing levels of petite concentrations. Similarly, the yeast in suspension behaviour and the change in wort density attenuation between the control and petite enriched fermentations were insignificantly different ($p > 0.05$). This study suggests that low concentrations of petites in the pitched yeast would not be detectable in the final product.

Key words: *Saccharomyces cerevisiae*, brewers yeast, petite mutation, respiratory deficient, fermentation, beer flavour

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Other than the funding of this research (see above) neither financial interest nor benefit has arisen from the direct application of this research.

Introduction

The most common mutation found in brewing yeast cultures is the respiratory deficient (RD) or 'petite' mutation [1]. Almost 70 years ago Ephrussi and colleagues [2] discovered this mutation. They are called 'petites' due to their small colony morphology when grown aerobically [2, 3]. There are two genotypes of petite mutations, *rho-* that lack large sections of mitochondrial DNA (mtDNA*]* or *rho0* that lack the entire mtDNA [4]. All petites are unable to grow on non-fermentable carbon sources [3]. In addition to this, previous research has demonstrated that petite mutated cells exhibit downregulated genes involved with late stages of the tricarboxylic acid (TCA) cycle [5].

Petites with the genotype *rho0* have been discussed in literature and it was only recently that the *rho0* mutation has been found naturally in brewing fermentations [6]. Research with flor yeasts has shown that the *rho*-mutation can change into complete loss of mtDNA over time [7]. The mutations are spontaneous, however, external factors may stress the cells and as a result may encourage the formation of the mutations.

The development of petites at different points during the brewing process has been studied [6, 8, 9]. In the yeast sediment found in the cone of the fermenter post fermentation, higher frequencies of petites were found at the same location as older cells [8]. The same study found that older cells are more susceptible to artificial petite mutation induction with ethidium bromide than virgin cells [8]. Petites can also form while the cells are being stored for re-pitching. The environment during storage is cold with limited nutrients available to the yeast. One study showed that as yeast culture storage time increased, the frequency of petites also increased and day three to four appears to be the crucial time when an increase in petite mutation frequency is observed [9].

High frequencies of petite mutants do not naturally exist in brewing yeast. One brewery reported that petite mutation frequency could range from 1 to 4 %, while another brewery encountered lower rates of less than 0.1 to 0.6 % by comparison [10]. However, there have been reports of higher levels when culture of yeast has been re-pitched. Jenkins and colleagues demonstrated that in one instance, as yeast was re-pitched up to fermentation (generation) eight, the concentration of petite mutants in the culture increased to 12 % for a lager strain of *Saccharomyces cerevisiae* (syn. *S. pastorianus* [9]). Another study showed that as a lager strain was re-pitched, the levels of petite mutations in the pitching yeast did not increase correspondingly and the highest frequency of petites reached was 0.55 % [6]. Both of these studies were done on industrial samples and the dissimilarity between the two studies was suspected to be due to yeast storage and handling stresses [6].

High levels of petite mutations present in fermentations cause problems [1, 11]. Van Zandycke and colleagues found that petite cells showed reduced flocculation using the improved Helm's test compared to its respiratory competent strain, resulting in aberrant fermentations [12]. RD yeasts were also shown to ferment at a slower rate than the same respiratory competent strain [11, 13, 14]. Several authors have found that RD cells cause off-flavours in the beer [1, 11, 14]. Šilhánková and colleagues investigated flavours in beer that were fermented with cultures of yeast where approximately 100 % of cells were mutated [14]. The results suggested the beer was more aromatic and contained lower levels of higher aliphatic alcohols [14]. Conversely, another study demonstrated that beer fermented with an RD strain produced more isobutanol and isoamyl alcohol in the beer than its parent strain [11]. The diacetyl levels were also higher in the beer produced with the RD strain [11]. Lastly, the results showed that the mutated strain did not accumulate as much biomass as the respiratory sufficient (RS) strain [11].

Publications in this field of research suggest that researchers are beginning to understand the complexity of how 100 % petite mutated cultures of yeast affect beer and fermentation; however, it is also important to understand the effect that petites have on fermentation and final product at the frequencies of their natural occurrence. Frequencies of 100% petite mutations have not been observed to exist in brewing fermentations (9, 10]. In fact, these published studies showed that with serial re-pitching the frequencies of petites have in only one instance reached 12 % with one strain, however no other yeast crops have not reported petite levels above 9 % [9]. Another study showed that the highest frequency of petites observed with one strain while serial re-pitched was 0.6 % [10].

This study investigated the extent to which petites contribute to the fermentation of beer and the flavours produced when beer is fermented with low levels of induced petite mutations in the inoculated yeast. Additionally, how low levels of petites impact the density attenuation and the yeast in suspension trends as the fermentation progressed were investigated.

Experimental

Yeast Propagation

Laboratory-scale fermentations were conducted with varying levels of petite mutations in the yeast cultures used for inoculation. For the control culture, lager yeast (*S. pastorianus*) strain SMA (VLB Berlin, DEU) was propagated from a YEPD slope (1 % w/v Yeast Extract, 2 % w/v Bacteriological Peptone, 2 % w/v D-Glucose anhydrous, and 1.5 % w/v Bacteriological Agar all from Fisher Scientific UK Limited, Loughborough, UK) into YEPD media (1 % w/v Yeast Extract, 2 % w/v Bacteriological Peptone, 2 % w/v D-Glucose anhydrous) using the propagation step in the miniature fermentation assay, Yeast-14 [15]. This resulted in 3.7 % petite mutations present in the yeast culture. Higher experimental petite levels were forced by

the addition of 20 µg/mL ethidium bromide solution (Sigma Aldrich Company Limited, Gillingham, UK) in the second propagation step of Yeast-14, and blended with a culture of yeast that was propagated according to Yeast-14 without ethidium bromide [15]. The concentration of ethidium bromide and incubation time was chosen based on the results from a preliminary study (see Supplementary Material). Ethidium bromide has been widely used for inducing the petite mutation [16, 17, 18, 19, 20]. The desired concentrations of petites were achieved by blending mutated yeast cultures and control cultures. The theoretical concentrations of petites for fermentation were 0 (control), 5, 10, and 15 %. Experimental concentrations of petite mutated cells in the culture achieved were 3.7 (control), 5.1, 8.7, and 10.8 % (see Supplementary Material) as determined by the 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Aldrich) overlay method [21]. Samples for this method were obtained at time 0 of the fermentation. This eliminated the error observed in the theoretical calculations. To mimic industrial fermentations where petite mutations spontaneously arise, each mutated cell was induced from the ethidium bromide in the media and not propagated from a single mutated cell. Cells were counted prior to pitching and during the re-propagation step in Yeast-14 [15]. For counting, the yeast slurry was diluted using 0.1 N sodium acetate trihydrate (Fisher Scientific) with 10 mM ethylene-diamine-tetraacetic acid (Sigma Aldrich) at pH 4.5. That solution was added 1:1 to a methylene blue solution (0.01 % w/v methylene blue and 2 % w/v sodium citrate tribasic dehydrate in distilled water) to analyse yeast viability. The cells were counted using a haemocytometer and the concentration determined as % viable cells.

Wort Preparation

A single batch of wort was prepared prior to the fermentations with sufficient volume to complete four fermentations using a 2 hL pilot plant (International Centre for Brewing and

Distilling, Edinburgh, UK). The grain bill consisted of 100 % extra pale malted barley (Crisp Malting, Fakenham, UK) and was milled using an Essex Major hammer mill (Christy Hunt Limited, Manchester, UK). Grain was added into the mash at a liquor: grist ratio of 2.8: 1. The initial mash temperature was held at 45 °C for 30 min., and then increased to 65 °C for 60 min. Wort separation was achieved using a Meura 2000 mash filter (Meura, Peruwelz, BEL). Pelleted Columbus hops (John I Hass Incorporated, Washington, DC) with an alpha acid content of 13.7 % were added at the start of the 60 min. boil to yield 20 International Bitterness Units (IBU). The original extract was 14.22 °P post-boil. The wort was distributed into 2 L containers and frozen (-20 °C). The wort was thawed at 4 °C for three days prior to use. Following this, the wort was autoclaved [121 °C, 101 kPa) for 15 min. and cooled before the fermentation started.

Fermentation

The fermentations were carried out using a modified version of the American Society of Brewing Chemists (ASBC) Yeast-14 procedure [15]. The wort was centrifuged at 4500 x g for 15 min. in sterile bottles in a fixed rotor centrifuge (Beckman Coulter Incorporated, Brea, CA), and the supernatant collected. Prior to pitching, the wort was adjusted to 12.6 °P with sterile distilled water and then 4% w/v D-glucose anhydrous (Fisher Scientific) was added, which achieved 14.7 °P. Additionally, the wort was oxygenated using sterile oxygen for five minutes. The oxygen was introduced into the wort by bubbling sterile oxygen through a 1 mL glass pipette while the wort was mixed vigorously with a magnetic stir bar. The yeast culture was pitched at 1.5 x 10^7 cells/mL into the wort to start fermentation. A volume of 20 mL of inoculated wort was decanted into test tubes (d=18 mm), yielding a fermentation height of 9 cm. Each fermentation batch contained 39 individual test tube fermenters. Throughout the fermentation, samples were taken in triplicate at set time points by destructive sampling for absorbance and density measurements. Absorbance was measured at 600 nm and 20 mL of

sample was filtered using a Whatman #4 filter paper (Sigma Aldrich) and the density (°P) and alcohol (% v/v) was measured with a DMA 4500 and an Alcolyzer beer ME system (Anton Paar, St Albans, UK).

At the end of each fermentation (78 hours), three fermenting tubes were centrifuged at 3000 x g for three minutes. The supernatant was frozen (-20 °C) and stored for later chemical analysis. Esters, higher alcohols, and vicinal diketones (VDKs) were analysed using headspace gas chromatography (GC). A Chromepack CP-Wax-57-CB column of dimensions 60 m x 0.25 mm x 0.40 μm (length, diameter, and film thickness respectively) was used with a Hewlett Packard 589-series II GC (Agilent Technologies, Stockport, UK) with split/splitless injector with a Flame Ionisation Detector (FID) and an Electron Capture Detector (ECD). The VDK's were measured with the ECD and the esters, higher alcohols and other flavour compounds analysed with FID. The VDK samples were preheated at 60 °C for 90 min. The internal standard was 200 mg/L 3-heptanone +18.1 mg/L hexanedione in absolute alcohol. Temperature settings for the oven, needle, and transfer tubing were 60, 70, and 110 °C respectively. The column flow rate was set to 1.5 mL/min.

Analysis

The absorbance readings were fitted to a tilted Gaussian fit according to the method of Yeast-14 [15] using Equation 1,

$$Abs_{600} = R \cdot t + A \cdot e^{-\frac{1}{2}\left(\frac{t-\mu}{\sigma}\right)^2} \quad \text{(Eq. 1)}$$

where Abs_{600} is the absorbance at any time t, R is the slope, μ is the midpoint, σ is the width factor, and A is the absolute amplitude [15]. An F-Test was used to determine whether the regression was significant ($p<0.05$).

The density attenuation was monitored with a four-parameter nonlinear regression using Equation 2 [15],

$$P_t = P_e + \frac{P_i - P_e}{1 + e^{-B(t-M)}} \quad \text{(Eq. 2)}$$

where P_t is the extract value at time t (°P), P_e is the final asymptotic extract value (°P), P_i is the initial asymptotic extract value (°P), B is a function of the slope at the time of inflection (°P/hr) and M is the time it takes to reach the inflection point (hr). An F-Test was performed on the individual parameters to determine whether the varying petite concentrations had an impact on the specific parameters of the fermentation. Additionally, an F-Test was performed on the regression.

The flavour compounds were analysed using linear regressions and an F-Test was performed to determine whether the slope was non-zero. A p-value < 0.05 was considered significant for all analyses. All statistical analyses were performed using Graphpad Prism (La Jolla, CA).

Results and Discussion

Yeast Viability

Before mixing the RD yeast culture with the RS yeast culture, the viabilities of each yeast culture were determined (See Table I). The results showed that the viability of all cultures propagated, both untreated and treated with ethidium bromide, remained above 98 %. It was expected that the ethidium bromide would not be detrimental to the viability of the propagated culture with ethidium bromide because similar results were found in a recent study [22]. The concentrations of ethidium bromide in this recent study were lower (0.5 μg/mL and 5 μg/mL), however, no significant differences in viabilities determined by cell forming units were found when compared to an untreated culture [22].

Flavour Compounds

Higher Alcohols

No significant differences ($p > 0.05$) were found between petite mutation levels and the presence of higher alcohols post-fermentation, specifically propan-1-ol, 3-methyl butanol, 2-methyl butanol, and isobutanol (Figure 1).

Previous studies examined the levels of higher alcohols in beer fermented with high levels (100 %) petite mutations in the pitched yeast. One showed increased levels of isobutanol, isoamyl alcohol, and propan-1-ol when fermenting with a 100 % RD culture in comparison to fermenting with its RS culture [11]. Morrison and Suggett also investigated flavour profiles post fermentation with a culture from a singular petite isolate compared its RS type [10]. The study found isobutanol and isoamyl alcohol levels increased however propan-1-ol levels decreased [10]. Contrary to both of those studies, another study investigated higher alcohols together and found decreased levels in fermentations with 100 % RD cultures [14]. The impact that high levels of petite mutations have on higher alcohol levels does not seem universal. The differences observed could be due to several factors such as yeast strain, petite mutation type, and/or wort composition. These studies were based on yeast cultures with approximately 100 % RD yeast.

The current study found with increasing levels of petites (< 10.8 %) in the pitching yeast the levels of propan-1-ol, isobutanol, 2-methyl butanol and 3-methyl butanol) were unchanged. The results from the current study suggests when petites are present in low concentrations, any over or underproduction of higher alcohols in fermentations that might exist [10, 11, 14], may be concealed by the normal variation in higher alcohol production between fermentations.

Esters

The esters analysed were ethyl acetate, isobutyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, and isoamyl acetate. All esters showed positive linear correlations with the increasing frequency of petite mutations in the pitching yeast (TableII). Of these positive linear correlations, ethyl acetate showed the largest increase related to the petite mutation frequency.

Ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate had strong or very strong linear correlations (r^2 = 0.690 – 0.955), and isobutyl acetate had a moderate positive correlation (r^2 = 0.430 – 0.442) as described by Evans [23]. An F-Test showed that the slope for these correlations were significantly ($p > 0.05$) non-zero. The positive linear correlations with increasing petite mutation concentration and isoamyl acetate, ethyl acetate, ethyl hexanoate, and ethyl octanoate are shown (Figure 2).

Two prior studies have investigated ester levels in beer fermented with high levels of petites [10, 11]. One of these studies showed the production of six esters analysed in beer fermented with a petite mutant strain were decreased when compared to beer fermented with its RS strain [10]. The other study found that two of the four ester concentrations analysed post fermentation were decreased for beer fermented with a petite mutant strain when compared to its RS type [11] while the other two esters were increased. Considering esters are influenced by several factors in fermentation, there may be several hypotheses for the discrepancy between the two published studies such as yeast strain, petite mutation type, wort composition, fermentation temperature, and/or wort aeration rates for example.

There are two groups of esters: acetate esters and medium chain fatty acid ethyl esters (MCFA). MCFA ethyl esters are formed by a condensation reaction with acyl-CoA and ethanol [24], and the enzymes involved in the synthesis of these are coded by Eeb1 and Eht1 genes [25]. The MCFA esters in the current study analysed were ethyl hexanoate and ethyl octanoate. The findings presented here for ethyl octanoate agree with a past study [11] that found an

increase in ethyl octanoate in the RD strain when compared to its RS strain, but not with another study who found a decrease in both ethyl octanoate and ethyl hexanoate [10].

The other group of esters, acetate esters, are anabolized by the enzyme alcohol acetyltransferase (AAT) coded by ATF genes anabolizes the condensation reaction between acetyl CoA and alcohol [24]. The acetate esters investigated in this study were ethyl acetate, isoamyl acetate, ethyl butyrate, and isobutyl acetate and all were found to increase with respect to increased petite mutations in the pitched yeast.

One plausible cause for observing the increase in acetate esters and MCFA ethyl esters could be from excess acetyl CoA due to disruption in other pathways that also utilize acetyl CoA. The compound acetyl CoA is utilized in the TCA cycle and is involved in the synthesis of lipids, nucleic acids, amino acids, and esters [26, 27]. Petites are unable to synthesize fatty acids because possession of functional mitochondria is necessary for lipid synthesis [1]. Additionally, in petite mutant cells, the TCA cycle genes are downregulated [5], thus potentially increasing the pool of acetyl CoA available for ester synthesis. Supporting this hypothesis, a past study showed that once lipid synthesis during normal fermentation ceases, acetate ester synthesis rates increase [28]. In petite mutated cells lipid synthesis is disrupted, and the higher levels of esters observed in the current study from fermentation with higher levels of petites could be a result of ester synthesis commencing earlier in fermentation for RD yeasts than its RS type.

Vicinal Diketones

Pentanedione and butanedione levels post fermentation increased with the increasing levels of RD yeast studied (Table II). Butanedione showed moderate positive correlations (r^2=0.423) and pentanedione showed weak positive correlations (r^2=0.347) as described by Evans [23]. An F-Test showed that the slope for these correlations were significantly ($p > 0.05$) non-zero. The samples for all flavour compounds were taken nearing the end of fermentation

at hour 78. Vicinal diketone levels nearing the end of fermentation are dynamic and any difference in fermentation progression between fermentations at hour 78 may contribute to some variation observed between samples. In general, published literature shows elevated vicinal diketone levels are associated when high levels (approximately 100 %) of petite mutations in the pitched yeast are present [10, 11].

Dasari and Kölling, provided a thorough study on petite mutants and their production of diacetyl in brewing [29]. These authors showed that the key enzyme (Ilv2) in α-acetolactate synthesis, which normally resides in the mitochondria, accumulated in the cytosol of RD yeasts [29]. Their data suggested that the enzyme accumulation in the cytosol was linked to the elevated levels of diacetyl produced by petites [29].

The current study contributes to the literature and shows that even with low levels of petites, butanedione and pentanedione levels for this yeast strain and petite mutation were increased. This demonstrates that with fermentations containing low levels of petite mutations, increased levels of vicinal diketones may be a concern.

Overall Impact

Flavour thresholds vary depending on several factors, such as the flavour compound, beer style and human sensitivity [30] making it difficult to determine when the increase in flavour compound would be noticeable in the beer. Even though in the experiment the levels of esters increased with increasing levels of petites in the pitched yeast, it does not necessarily indicate that low levels of petites would be detectible to the consumer. From the results, the differences in ester and VDK concentrations produced from 0 to 5 % increase and 0 to 10 % increase of petites in the experiment were calculated (Table III) and compared to published flavour thresholds for the compounds [27]. Viewing this table, it is unlikely that the change in

flavour compounds produced would be detectible to the consumer because they do not surpass the documented flavour thresholds [27].

Alcohol Production during Fermentation

The ABV was monitored through the fermentation (Figure 3). A one-way ANOVA showed that there was no significant difference ($p < 0.05$) for the four fermentations at hour 78. This time point was chosen because it was the same time that samples were taken for flavour analysis. Similar to the higher alcohol levels with varying levels of petites in the pitched yeast, the ABV values were unchanged. This showed that the low levels of petites present (< 10.8 %) had no impact on the final alcohol levels at the end of fermentation.

Yeast in Suspension during Fermentation

The absorbance was measured periodically during fermentation as a representation of the yeast in suspension (Figure 4]. Results showed no significant difference ($p < 0.05$) in yeast in suspension behaviour at the levels of petite mutated yeasts tested.

Ernandes and colleagues [11] and Šilhánková and colleagues [14] demonstrated that during fermentation, a respiratory deficient strain produced less biomass compared its RS strain. This trend was not observed to be consistent in the experimental data presented. The small scale fermentations undertaken demonstrated no significant difference ($p > 0.05$) between the four fermentations with respect to the yeast in suspension profile. This result was unexpected as the petite mutant is considered a weak competitor to the RS strain, due to slower growth [13]. Therefore, one might expect a decreased absorbance peak with respect to yeast in suspension trends as petites levels increased during the fermentation. This was not observed. The majority of the cells present in fermentation were RS with a small percentage of RD cells. Even though petite mutations exhibit slower growth, the normal fermenting capacity of the normal cells in fermentation outweighed the petite mutations. In the work presented, a petite

population of up to 10.8 % did not impact the overall yeast growth in fermentations. This was proposed to be from the slower growth of the respiratory deficient strain, therefore leaving more nutrients available for growth of the RS strain. As RS cells grow quicker than the RD cells during fermentation, a decrease in the frequency of petites might be expected with serial re-pitching. In one previously published study that investigated serial re-pitching and the frequency of petites, the highest frequency of petites was not observed with the highest re-pitched fermentation [6]. This may be observed in specific cases, however the susceptibility of a strain to produce its petite mutant is strain specific. While serial re-pitching, the yeast culture is repeatedly exposed to the stresses in the cone. A previously published study reported that a yeast culture that had been serial re-pitched was more susceptible to petite mutation formation, when subjecting the different populations to ethidium bromide [9]. This suggests that the accumulation of petites with serial re-pitching observed is caused from an increase in mutation formation during yeast storage and handling, and not from an accumulation during fermentation.

Furthermore, the yeast in suspension trends towards the end of the fermentation reflects the flocculation trends of the yeast in the fermentation. The Yeast-14 method was originally designed to investigate whether or not malt was susceptible to premature yeast flocculation [15, 31]. This was done by using the absorbance (600 nm) to determine flocculation patterns of the yeast during fermentation. The modelled yeast in suspension trends were insignificant ($p > 0.05$). This suggests that the flocculation trends between the four fermentations were not affected by the presence of petites from 3.7 % up to 10.8 %. The lack of impact on fermentation profile could be considered to be surprising given that the literature shows that petites have reduced flocculation patterns [12], however these results suggest those effects are not apparent on the yeast in suspension profiles when present at the low frequencies.

A large fraction of the total yeast at the end of fermentation was found to be still in suspension. A possibility may be that post fermentation, the percentage of petites could become a significant factor in the yeast in suspension values. To gain more of an understanding on the flocculation trends in fermentations with low levels of petites, the yeast in suspension would need to be monitored past the time it took for the fermentation to reach its final extract and into conditioning. However, the yeast in suspension trends shown demonstrated that the physical flocculation trends in the primary fermentation are not impacted by low levels of petite mutations in the pitched culture.

Density Attenuation during Fermentation

For the four fermentations completed, the only statistically significant density attenuation regression ($p > 0.05$) was the fermentation with the lowest frequency of petites [3.7 %) whereas the other fermentations with 5.1 %, 8.4 %, and 10.8 % RD yeasts showed identical attenuation trends. Additionally, three parameters of the density attenuation model of Equation 2 (M, B, and P_e) were not significantly different ($p<0.05$) for all four fermentations. These parameters demonstrated that low levels of petites in the pitched yeast had no impact on the speed of fermentation, the midpoint of the fermentation, and final density.

Previously published literature illustrated that fermentations with petite mutated cells fermented slower than their parent strain [11, 13]. In the experimental work presented here, the impact that high frequencies of petites have on density attenuation was determined to be undetectable when low frequencies of petites are present. Even though petite mutations ferment at a slower rate than their grande-type, when the petite mutants are present in low quantities, no difference in fermentation speed (B) or time for the fermentation to reach the midpoint (M) was detectible. Two previously published studies [11, 13] both found that the petite strains did not reach final gravity equal to its respiratory competent genotype. In the current study, the end

gravity (P_e) was insignificant between all fermentations with low levels of petites including the control. This showed that low levels of petite mutations did not have an impact on the different fermentation parameters: final density, the midpoint, and speed of fermentation.

Conclusions

Petite mutations are a complex phenomenon; thus, this complexity makes it difficult to compare between studies and make general claims. Differences in strain, the specific deletion in *rho-* mutations, and fermentation conditions create a handful of variations that may have caused a multitude of outcomes between studies, many of which have been highlighted here.

While it is appreciated that the petite mutations in this experiment were not spontaneously produced as seen in the brewing industry, the method used to induce the RD mutation is a widely accepted method for petite induction [17, 19, 22, 32, 33] and therefore would give an indicator to how spontaneously produced mutations might react in fermentation. In fact, the levels of butanedione that ethidium bromide induced petites produced during fermentation has been previously studied [29].

Interestingly from the varying levels of mutated cells in the experimental fermentations, the higher alcohols were not affected suggesting that any under or overproduction of higher alcohols that was found in previous studies [11, 14] was undetected at low concentrations of petites used in this study. Variation in higher alcohol levels produced between normal fermentations exists and it seems any impact that petite mutations may have on these levels is hidden in the fermentation variation. The results also demonstrated that the concentration of esters and VDKs were positively correlated to an increase in petite mutations in the fermentation, with some esters having a stronger correlation than others. However, it could be

argued that consumer detection of changes in flavour compounds between fermentation with varying levels of petites is unlikely.

The yeast in suspension trends were not significantly impacted by the low levels of petite mutations, suggesting that the overall flocculation of the culture used for fermentation was not impacted with low levels of petites present. In published literature, however, a study showed that flocculation properties of high levels of petites were altered [12]. It was speculated that the difference in results between studies was due to different concentrations of petite mutations in the cultures. The previously published experiments argued that flocculation properties were impacted with **very** high levels of petite mutated cells present. However, this experiment suggests that with **low** levels of petite mutated cells, of the level present in industrial fermentations little effects occur.

Furthermore, the parameters in the density attenuation regression related to the midpoint, speed of fermentation, and final gravity of fermentation were unchanged between each frequency of RD yeast in the pitched yeast studied.

While the results found here indicated that the effects of petites were undetected in low concentrations, this provides an insight into the behaviour of petites in industry size fermentations. On an industrial scale, the mass of yeast pitched into fermentation is much larger, and the increased mass may react differently. Furthermore, some strains of yeast may tolerate petite mutations better than other strains.

It is important to consider that prior to cells exhibiting a petite mutation phenotype in any fermentation, degradation of mtDNA starts. This is particularly observed when serial re-pitching. By studying fermentations with ethidium bromide induced petite mutations, any effect that these cells pose on fermentation could not be observed.

Petite mutations are a concern in the brewing industry because they have altered flocculation characteristics [12], accumulate less biomass [11], and overproduce and/or under

produce flavour compounds [10, 11, 14]. However, considering all the parameters monitored during this current study, it is unlikely that low levels (< 10.8 %) of the petite mutated cells present in the pitched yeast are undetectable in the fermentation profile and final product for the yeast strain SMA.

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List of Tables

List of Figures

Tables

Table I. Viabilities of yeast cultures used after propagation and prior to blending for pitching. Means represent duplicate counts with standard deviation.

	0		5		10		20	
	Avg. (%)	SD	Avg (%)	SD	Avg (%)	SD	Avg (%)	SD
Grandes	99.7	0.01	99.6	0.2	99.5	0.3	99.6	0.6
Petites	ND	ND	98.4	0.2	99.4	0.2	98.6	0.4

Table II. Summary of flavour compounds analysed by linear regression as the percentage of petite mutation in the pitching yeast increased from 3.7 % to 10.8 %. The table includes: the slope (m), y-intercept (b), coefficient of determination (r^2).

Flavour Compound	Slope (m)	Y intercept (b)	r^2	p	Deviation from zero ($p < 0.05$)
Acetone	0.001104	0.7522	0.02977	0.5918	Not Significant
Ethyl acetate	0.5597	12.39	0.6895	0.0008	Significant
Isobutyl acetate	0.001563	0.03119	0.4421	0.0183	Significant
Ethyl butyrate	0.002155	0.04086	0.9552	< 0.0001	Significant
Propan-1-ol	0.0002408	32.19	8.5E-08	0.9993	Not Significant
Isobutanol	0.03515	17.79	0.00768	0.7866	Not Significant
Iso amyl acetate	0.05882	1.051	0.844	< 0.0001	Significant
2-Methyl butanol	0.0539	19.46	0.02397	0.6309	Not Significant
3-Methyl butanol	-0.006955	58.07	2.8E-05	0.9869	Not Significant
Ethyl hexanoate	0.004165	0.07743	0.8291	< 0.0001	Significant
Ethyl octanoate	0.01449	0.04193	0.9449	< 0.0001	Significant
Butanedione	0.005108	0.07111	0.4296	0.0207	Significant
Pentanedione	0.00687	0.1116	0.3465	0.0441	Significant

N.B. n=12.

Table III. Using the linear regression results for each flavour compound reported in Table 2, the increase between 0 and 5 % petites, and 0 and 10 % petites were calculated. These values were compared to the published flavour thresholds for the flavour compound in beer [27].

Flavour Compound	Δ [flavour compound] (mg/L) from 0 % and 5 % petites	Δ (flavour compound] (mg/L) from 0 % and 10 % petites	Threshold (mg/L) [24]
Ethyl acetate	2.80	5.60	25-30
Isobutyl acetate	0.0078	0.016	n.d.*
Ethyl butyrate	0.011	0.022	n.d.*
Iso amyl acetate	0.29	0.59	1.2-2.0
Ethyl hexanoate	0.021	0.042	0.2-0.23
Ethyl octanoate	0.072	0.15	0.9-1.0
Butanedione	0.026	0.051	0.1-0.15
Pentanedione	0.034	0.069	0.9-1.0

*n.d. signifies no data available.

580

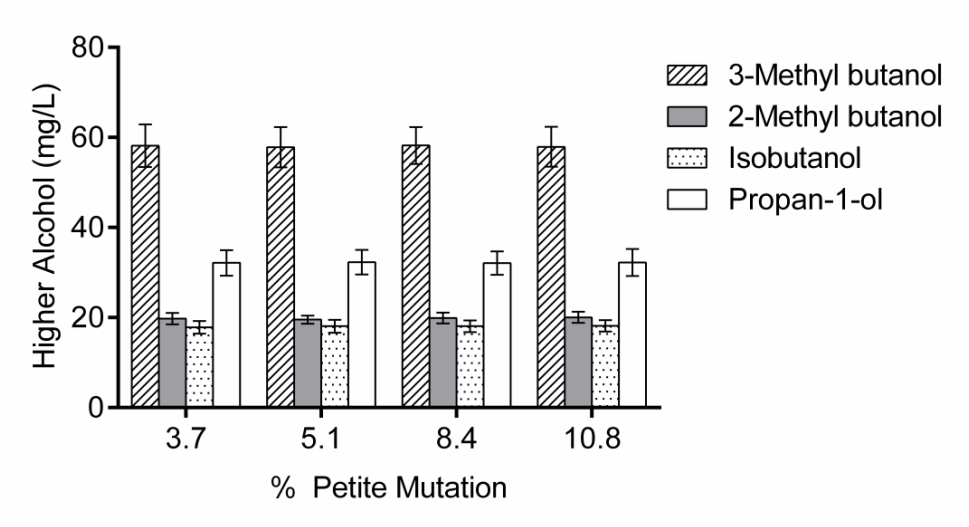
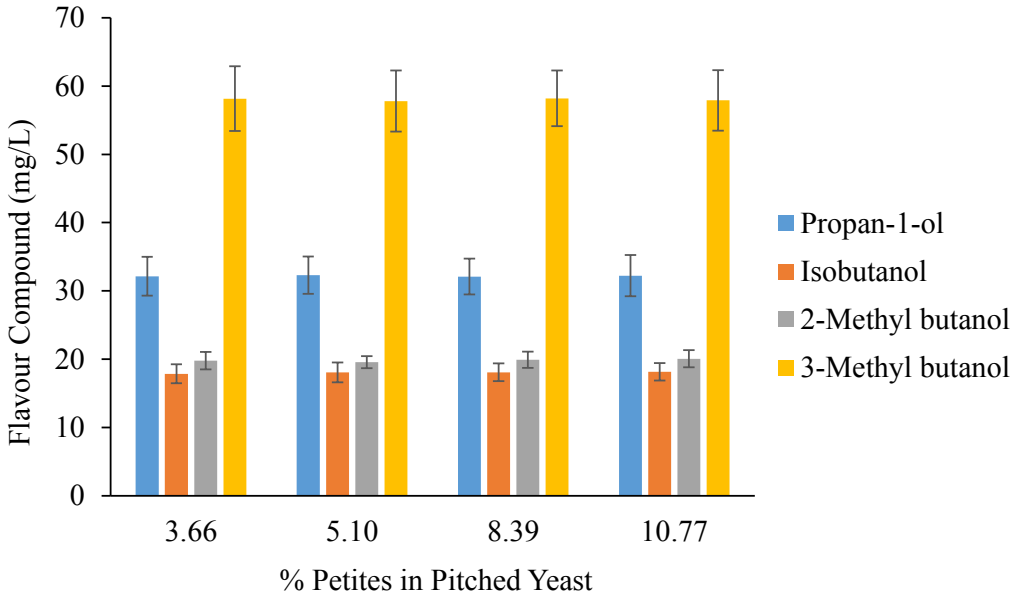

Higher Alcohol (mg/L)
80
60
40
20
0
3.7
5.1
8.4
10.8
% Petite Mutation
3-Methyl butanol
2-Methyl butanol
Isobutanol
Propan-1-ol

Figure 1.


Flavour Compound (mg/L)
70
60
50
40
30
20
10
0
3.66
5.10
8.39
10.77
% Petites in Pitched Yeast
Propan-1-ol
Isobutanol
2-Methyl butanol
3-Methyl butanol

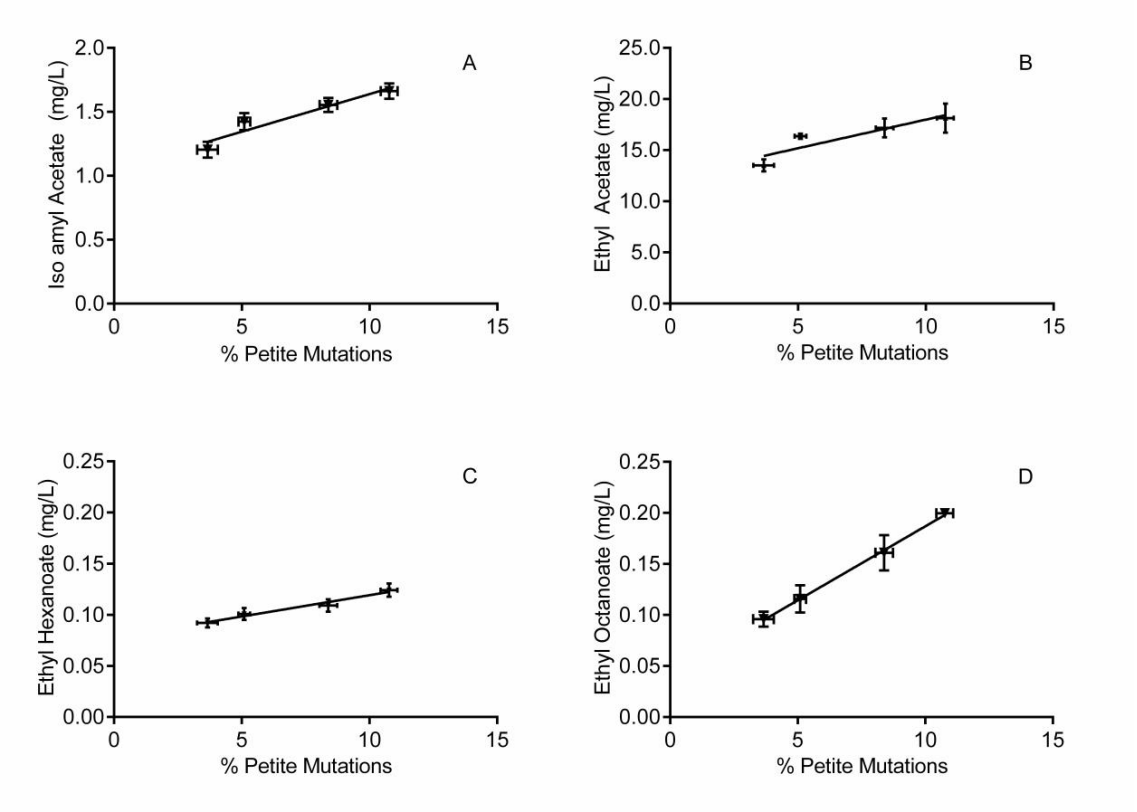

A
Iso amyl Acetate (mg/L)
2.0
1.5
1.0
0.5
0.0
0
5
10
15
% Petite Mutations
B
Ethyl Acetate (mg/L)
25.0
20.0
15.0
10.0
5.0
0.0
0
5
10
15
% Petite Mutations
C
Ethyl Hexanoate (mg/L)
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
% Petite Mutations
D
Ethyl Octanoate (mg/L)
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
% Petite Mutations

Figure 2.

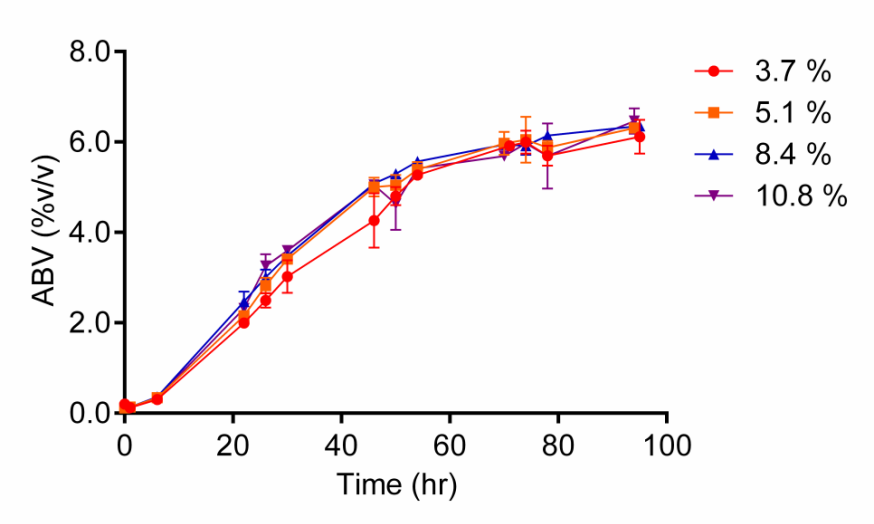


Figure 3.

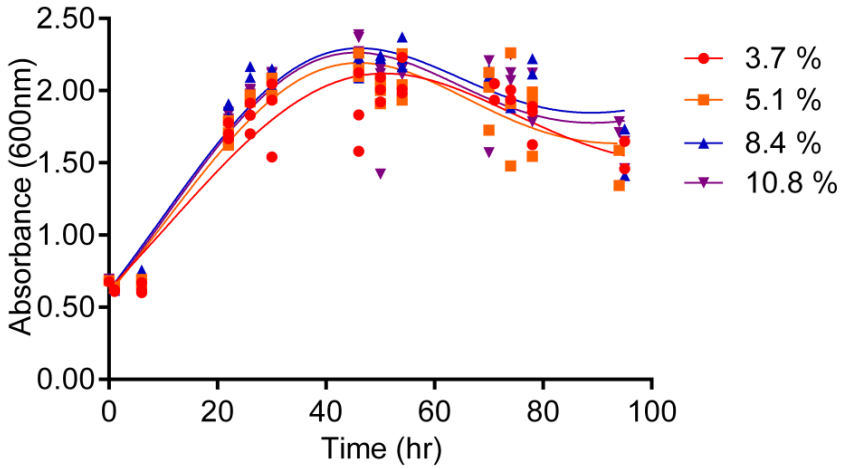

2.50
2.00
1.50
1.00
0.50
0.00
Absorbance (600nm)
0
20
40
60
80
100
Time (hr)
3.7 %
5.1 %
8.4 %
10.8 %

Figure 4.

Supplementary Material A – Preliminary Experiment

Methods

A preliminary experiment was completed to determine the mutation rate of the two different yeast strains (SMA and W34/70) when exposed to varying concentrations of ethidium bromide. A loopful of yeast was taken from a YEPD agar slope and inoculated into 50 mL of YEPD broth. The yeast was cultured for 24 hours in a shaking incubator (30 °C, 100 rpm). The yeast was centrifuged to pellet, supernatant discarded and the pellet was suspended in distilled water three times. The yeast was pitched at 1.5E7 cells/mL into YEPD broth with varying levels of ethidium bromide concentrations for different lengths of time (Table 5). Additionally, the same treatments with static incubations was attempted. However, the yeast formed large flocs that would not break apart in the presence of EDTA. The large flocs caused inaccurate cell counts for pitching. Once the treatment was completed the petite mutation frequency was determined by a dual plating method with glycerol and glucose

Results

Table 5. Preliminary experiment to determine the petite mutation rates of strains SMA and W34/70. Cells were incubated at stated concentration of ethidium bromide in YEPD broth at 30 °C and 100 rpm. After the stated time interval the cells were plated onto YEPD and YEPGLy plates to determinethe percentage of petites.

Yeast Strain	EtBr Treatment (μg/mL)	Incubation time (h)	Cell count YEPD	YPGly	% Petites
SMA	10	1	389	379	3
		4	TNTC*	TNTC	ND**
		24	398	237	40
SMA	20	1	708	628	11
		4	TNTC	TNTC	ND
		24	441	34	92
W34/70	10	1	688	704	-2
		4	TNTC	TNTC	ND
		24	156	122	22
W34/70	20	1	620	652	-5
		4	TNTC	TNTC	ND
		24	558	98	82

*TMTC = Too numerous to count

**ND = No data

Discussion and Conclusion

Incubating the cells with 10 and 20 μg/mL of ethidium bromide for one hour was not sufficient for mutating the cells. At four hours, there were too many cells to count the plates. However, under visual inspection, both plates showed similar growth suggesting that four

hours was too short for induction of petite mutations in these conditions. A large frequency of petites was induced with both strains when propagated with 20 μg/mL of ethidium bromide for 24 hours. Strain SMA showed the larger susceptibility to induction and was utilized for the primary study.

Supplementary Material B – TTC Overlay Technique Results

Fermentation	Replicate	Total Petites	Total Cells Counted	% Petites	Average	Standard Deviation
1	1	71	648	10.96	**10.7**	**1.2**
	2	57	645	8.84		
	3	66	680	9.71		
	4	65	635	10.24		
	5	52	576	9.03		
	6	58	546	10.62		
	7	79	652	12.12		
	8	81	650	12.46		
	9	61	549	11.11		
	10	63	548	11.50		
2	1	42	705	5.96	**5.1**	**0.7**
	2	44	736	5.98		
	3	39	711	5.49		
	4	34	707	4.81		
	5	39	766	5.09		
	6	36	801	4.49		
	7	41	748	5.48		
	8	36	645	5.58		
	9	26	624	4.17		
	10	26	664	3.92		
3	1	68	646	10.53	**8.4**	**1.1**
	2	46	593	7.76		
	3	41	614	6.68		
	4	51	603	8.46		
	5	45	621	7.25		
	6	51	600	8.50		
	7	65	686	9.48		
	8	63	706	8.92		
	9	52	621	8.37		
	10	51	639	7.98		
4	1	31	586	5.29	**3.7**	**1.0**
	2	27	588	4.59		
	3	17	596	2.85		
	4	16	534	3.00		
	5	29	622	4.66		
	6	18	620	2.90		
	7	26	561	4.63		
	8	11	454	2.42		
	9	14	536	2.61		
	10	18	490	3.67		